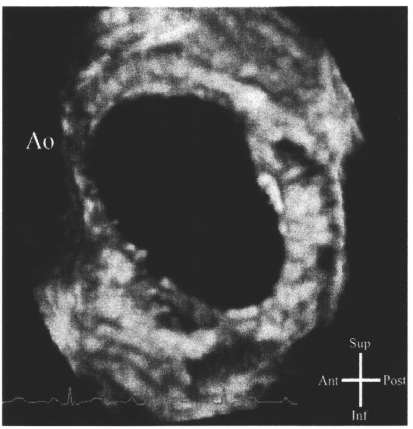


Figure 2 RT3D TEE images 3 months after the first procedure.

a solid rim, an aneurismal rim, and a fenestrated rim.[8] A detailed evaluation of the surrounding rims, including these tissue morphologies, should be performed before the procedure using 2D TEE with adequate temporal and spatial resolution.

In the present case, imaging during the procedure played a pivotal role to evaluate the situation and determine the operative strategy. 2D TEE and intracardiac echocardiography are mainly used for guidance of transcatheter closure of ASDs. Although these 2D imaging modalities are sufficient to guide the procedure in cases with simple ASD morphology, in cases with complicated ASD morphology, such as multiple defects or our present case, it is often difficult to reconstruct the spatial structure with the use of 2D images. In a previous case report, the torn rim was confused with the intracardiac thrombus by 2D TEE during the procedure.[3]

Previous studies have demonstrated the usefulness of 3D TEE reconstruction in transcatheter closure of ASDs.[9] Although 3D TEE reconstruction demands a complex acquisition and lengthy data analysis, RT3D TEE has become available in clinical practice, and it has been used in the catheterization laboratory and intraoperative setting.[5-7] We reported previously that RT3D TEE was a feasible and useful complementary option to 2D TEE for morphologic evaluation of ASDs and guidance of transcatheter closure of ASDs.[7] In our case, RT3D TEE demonstrated the torn rim by easily viewed en face images during the course of the procedure, which allowed us to choose an appropriate therapeutic strategy. Thin portions of the septum were visualized by 2D TEE. Areas of potential drop-out may be related to areas of thin septum or septal fenestrations, which should be investigated carefully using 2D TEE (because of its higher spatial resolution) and color Doppler ultrasound to determine whether true defects are present.

## CONCLUSIONS

We have reported a case with a rare complication of a torn rim during transcatheter closure of an ASD. RT3D TEE is useful for displaying the entire shape of the defect and its spatial relationship with its neighboring structures compared with conventional 2D echocardiography, and 2D TEE with adequate spatial resolution shows detailed information of the rim tissue. By using both 2D and RT3D TEE, especially in cases with complicated ASD morphology, both the echocardiologist and interventionalist gain valuable information on the morphology of the ASD before and after the procedure.

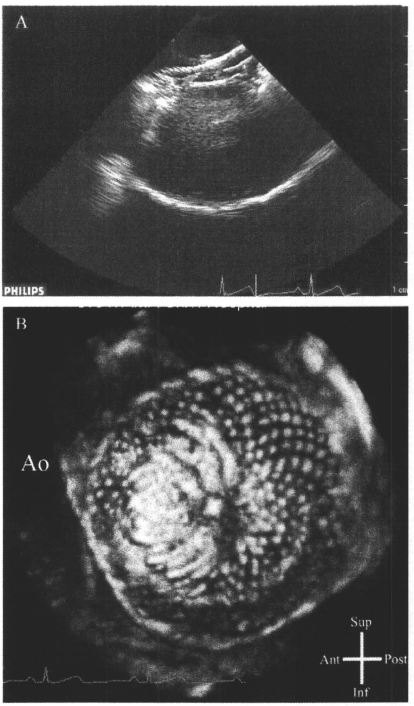


Figure 3 2D TEE **(A)** and RT3D TEE **(B)** images after deployment of ASO. The defect was successfully closed with a 32-mm ASO.

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# Pro-apoptotic effects of imatinib on PDGF-stimulated pulmonary artery smooth muscle cells from patients with idiopathic pulmonary arterial hypertension☆

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ABSTRACT

*Background:* Remodeling of the pulmonary artery by an inappropriate increase of pulmonary artery smooth muscle cells (PASMCs) is problematic in the treatment of idiopathic pulmonary arterial hypertension (IPAH). Effective treatment that achieves reverse remodeling is required. The aim of this study was to assess the pro-apoptotic effects of imatinib, a platelet-derived growth factor (PDGF)-receptor tyrosine kinase inhibitor, on PASMCs obtained from patients with IPAH.

*Methods:* PASMCs were obtained from 8 patients with IPAH undergoing lung transplantation. Cellular proliferation was assessed by $^{3}$H-thymidine incorporation. Pro-apoptotic effects of imatinib were examined using TUNEL and caspase-3,7 assays and using transmission electron microscopy.

*Results:* Treatment with imatinib (0.1 to 10 μg/mL) significantly inhibited PDGF-BB (10 ng/mL)-induced proliferation of PASMCs from IPAH patients. Imatinib (1 μg/mL) did not induce apoptosis in quiescent IPAH-PASMCs, but it had a pro-apoptotic effect on IPAH-PASMCs stimulated with PDGF-BB. Imatinib did not induce apoptosis in normal control PASMCs with or without PDGF-BB stimulation. PDGF-BB induced phosphorylation of Akt at 15 min, and Akt phosphorylation was inhibited by imatinib in IPAH-PASMCs. Akt-I-1/2 (1 μmol/L), an Akt inhibitor, in the presence of PDGF-BB significantly increased apoptotic cells compared with the control condition. Thus, Akt-I-1/2 could mimic the effects of imatinib on PASMCs.

*Conclusion:* Imatinib has anti-proliferative and pro-apoptotic effects on IPAH-PASMCs stimulated with PDGF. The inhibitory effect of imatinib on Akt phosphorylation induced by PDGF plays an important role in the pro-apoptotic effect.

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## 1. Introduction

Idiopathic pulmonary arterial hypertension (IPAH) is a progressive disease characterized by progressive elevation of pulmonary vascular resistance and pulmonary artery pressure. Increased pulmonary vascular resistance is induced by pulmonary vasoconstriction, vascular remodeling by intimal and medial hypertrophy, and thrombosis [1,2]. Pulmonary vascular medial hypertrophy is caused by an inappropriate increase in pulmonary artery smooth muscle cells (PASMCs). Treatment with several vasodilators such as calcium channel blockers, prostaglandin $I_2$ and endothelin receptor antagonists was found to improve survival of patients with IPAH, but 5-year survival remains at 50% [3,4]. Effective treatment that achieves reverse remodeling is needed. This will require anti-proliferative and pro-apoptotic agents for PASMCs.

We have reported that platelet-derived growth factor (PDGF)-BB stimulation causes a higher growth rate of cultured PASMCs from patients with IPAH than that of control cells [5–7]. Recently, the use of a PDGF-receptor inhibitor such as imatinib (STI571) is starting to garner attention as a targeted therapy for pulmonary hypertension (PH) [8–11]. Imatinib is a drug used to treat certain types of cancer such as chronic myelogenous leukemia and gastrointestinal stromal tumors. In laboratory settings, imatinib is used as an experimental agent to suppress PDGF by inhibiting PDGF receptor ß (PDGF-Rß). It is an agent that acts by specifically inhibiting a certain enzyme, tyrosine kinase, that

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**Table 1**
Clinical data of patients with IPAH.

| Patient | Time | Sex | Age | PAP (s/d/m) (mmHg) | mRAP (mmHg) | CI (L/min/m$^2$) | PVR (dyn/s/cm$^5$) | BNP (pg/dL) |
|---|---|---|---|---|---|---|---|---|
| 1 | Prior to drug therapy | F | 7 | 150/72/98 | 4 | 3.8 | 1918 | 136 |
| | Prior to transplantation | | 13 | 99/59/72 | 15 | 2.3 | 2779 | 334 |
| 2 | Prior to drug therapy | F | 28 | 88/40/59 | 10 | 1.9 | 1416 | 408 |
| | Prior to transplantation | | 31 | 73/30/48 | 1 | 2.1 | 1199 | 325 |
| 3 | Prior to drug therapy | F | 10 | 118/67/84 | 14 | 2 | NA | NA |
| | Prior to transplantation | | 13 | 111/49/67 | 10 | 1.7 | 2438 | 203 |
| 4 | Prior to drug therapy | F | NA | NA | NA | NA | NA | NA |
| | Prior to transplantation | | 28 | 113/36/66 | 7 | 1.8 | 3340 | 50 |
| 5 | Prior to drug therapy | M | 16 | 163/71/106 | 2 | 1.7 | 2267 | 14 |
| | Prior to transplantation | | 20 | 70/40/50 | 2 | 3.3 | 808 | 18 |
| 6 | Prior to drug therapy | F | 39 | 74/23/42 | 3 | 2.6 | NA | NA |
| | Prior to transplantation | | 43 | 107/47/72 | 15 | 2.4 | 3056 | 622 |
| 7 | Prior to drug therapy | F | 13 | 96/50/68 | 4 | 2.3 | 1495 | 411 |
| | Prior to transplantation | | 16 | 83/51/65 | 8 | 2.5 | 784 | 216 |
| 8 | Prior to drug therapy | M | NA | NA | NA | NA | NA | NA |
| | Prior to transplantation | | 11 | 130/51/80 | 9 | 1.9 | 2629 | 420 |
| Mean ± SE | Prior to drug therapy | | 19 ± 5 | mPAP: 76 ± 10 | 6 ± 2 | 2.4 ± 0.3 | 1774 ± 198 | 242 ± 100 |
| | Prior to transplantation | | 22 ± 4 | mPAP: 65 ± 4 | 8 ± 2 | 2.3 ± 0.2 | 2129 ± 366 | 273 ± 70 |

M: male, F: female, PAP: pulmonary artery pressure, s/d/m: systolic/diastolic/mean, mRAP: mean right atrial pressure, CI: cardiac index, PVR: pulmonary vascular resistance, BNP: plasma concentration of brain natriuretic peptide, NA: not available.

is characteristic of a particular cancer cell, rather than non-specifically inhibiting the proliferation of and killing all rapidly dividing cells. Schermuly et al. reported that imatinib reverses pulmonary vascular remodeling and cor pulmonale in rats with monocrotaline-induced PH and in mice with chronic hypoxia-induced PH [8]. Perros et al. reported that PDGF-BB-induced proliferation and migration of PASMCs from patients with IPAH were inhibited by imatinib [10].

Not only inhibition of proliferation but also induction of apoptosis of PASMCs is needed to actively reduce stenosis due to vascular remodeling at small pulmonary arteries of patients with IPAH. These two effects may lead to reverse remodeling of the pulmonary vasculature. Expression of PDGF-B is up-regulated in the medial layer of small pulmonary arteries of rats with monocrotaline-induced PH and imatinib induces apoptosis in the small pulmonary arteries [8]. However, imatinib does not induce apoptosis in cultured IPAH-PASMCs without PDGF treatment [10]. Thus, imatinib may not be able to induce apoptosis in quiescent cells. We hypothesized that imatinib in the presence of PDGF-BB induces apoptosis of PASMCs from patients with IPAH, but that imatinib cannot induce apoptosis in PASMCs without PDGF stimulation. We therefore investigated whether imatinib in the presence and absence of PDGF-BB induces apoptosis of PASMCs from patients with IPAH.

Akt is a member of the serine/threonine-specific kinase family known for facilitating cell survival via the inhibition of apoptotic pathways [12]. Therefore, induction of apoptosis of IPAH-PASMCs may be related to Akt inactivation. We also investigated whether imatinib inhibits Akt activation.

## 2. Materials and methods

### 2.1. Isolation, culture and identification of PASMCs

Peripheral segments of the pulmonary artery were obtained at lung transplantation [13] from 8 patients with IPAH as previously described [5,6,14,15] (2 males and 6 females; mean age, 22 ± 4 years; age range 11–43 years) (Table 1). For normal control experiments, samples of pulmonary arteries were also obtained at lung lobectomy from a patient with bronchogenic carcinoma (male, 58 years old) who showed no evidence of PAH and received no systemic chemotherapy or radiation therapy before lung lobectomy as previously described [5,6,14,15]. Samples of the pulmonary arteries were obtained from the most distal area from the carcinoma in the resected lobe. All of the studies were approved by the Ethics Committee of Okayama University Graduate School of Medicine, Density, and Pharmaceutical Sciences, and written informed consent was obtained from all patients before the procedure. The investigation also conforms to the principles outlined in the Declaration of Helsinki.

PASMCs were isolated as described previously [5,6,14–16]. Peripheral segments of pulmonary arteries smaller than 1 mm in outer diameter were disaggregated with collagenase and cut into 2-mm-long sections, and then the adventitia and endothelial cell layers were removed. Vessels were plated on a 6-well plate with Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Sigma) and 0.1 mg/mL kanamycin (Sigma) and incubated in a humidified 5% CO2 atmosphere at 37 °C. The culture medium was changed every 3 days. After reaching confluence, the cells were subcultured by treatment with trypsin

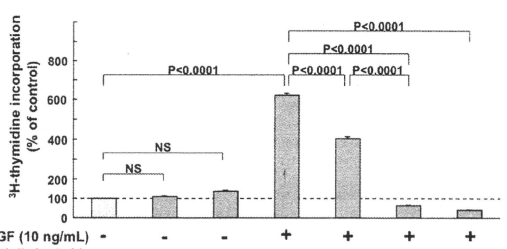


**Fig. 1.** Inhibitory effect of imatinib on proliferation of PASMCs from IPAH patients. Anti-proliferative effects of imatinib (0.1 to 10 μg/mL) on IPAH-PASMCs stimulated with PDGF-BB (10 ng/mL). $^3$H-thymidine incorporation was measured. Counts per minute (cpm) were expressed as a percentage of cpm of IPAH-PASMCs treated with a diluent (control). Data are mean ± SE.

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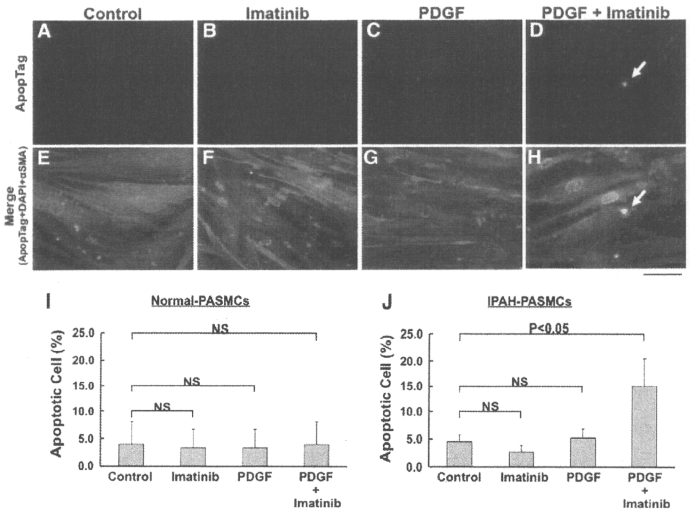


**Fig. 2.** Effect of imatinib on apoptosis of PASMCs in TUNEL assay by ApopTag fluorescein. A to D, ApopTag fluorescein (green). E to H, Combined images (merge) of ApopTag fluorescein, DAPI (blue) and αSMA (red). A and E, IPAH-PASMCs without treatment. B and F, IPAH-PASMCs treated with imatinib (1 μg/mL). C and G, IPAH-PASMCs treated with PDGF-BB (10 ng/mL). D and H, IPAH-PASMCs treated with imatinib and PDGF-BB. Arrow shows a TUNEL-positive cell (green). Bar = 500 μm. I, Effect of imatinib on apoptosis of normal PASMCs in TUNEL assay. J, Effect of imatinib on apoptosis of IPAH-PASMCs in TUNEL assay. Imatinib (1 μg/mL) in the presence of PDGF-BB (10 ng/mL) significantly increased TUNEL-positive (apoptotic) cells in IPAH-PASMCs compared with the control condition ($P<0.05$). Data are mean ± SE.

(0.05%)/ethylenediaminetetraacetic acid (EDTA) (0.02%). Cell identification was confirmed by the examination of cytoskeletal components (α-smooth muscle actin, myosin, and smoothelin) using an immunocytochemical technique as described previously [5,15]. Cells between passages 3 to 5 were used for all experiments.

### 2.2. Effects of imatinib on cell proliferation

To assess the antiproliferative effect of imatinib on PASMCs, we measured $^3$H-thymidine incorporation using methods described previously [5,16]. PASMCs were reseeded in 24-well plates at a density of $5\times10^4$ cells/well on day 0. After 16 h of incubation (on day 1), the culture media were replaced with low-serum culture media (DMEM, 0.1% FBS, and 0.1 mg/mL kanamycin), and the cultured cells were made quiescent for 48 h. On day 3, PDGF-BB (10 ng/mL) (Sigma), imatinib (0.1 to 10 μg/mL) (Novartis) or an Akt inhibitor, Akt-I-1/2 (1 μmol/L) (Calbiochem), was added to the media. After 21 h (on day 4), the cells were labeled with $^3$H-thymidine at 1 μCi/mL for 3 h. After completion of labeling, the cells were washed with ice-cold PBS, fixed with 5% trichloroacetic acid and 95% ethanol, and lysed with 200 μL/well of 0.33 mol/L NaOH. Aliquots of the cell lysates were neutralized with 1 mol/L HCl, and the radioactivity was measured in a liquid scintillation analyzer (TRI-CARB 2200CA; Packard, Downers Grove, IL, USA).

### 2.3. Western blot analysis

PASMCs from patients with IPAH were prepared in the same manner as that described for analysis of DNA synthesis. They were treated in the presence or absence of PDGF-BB (10 or 100 ng/mL), imatinib (1 or 10 μg/mL) and a mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) inhibitor, U0126 (3 μmol/L) (Promega). Western blot analysis was performed as described previously [5,7]. Briefly, total cell lysates of cultured PASMCs were extracted in commonly used radioimmunoprecipitation (RIPA) buffer with 10 mg/mL phenylmethylsulfonyl fluoride (Sigma) and then concentrated by centrifugation at 12,000 rpm for 20 min. Protein samples (10 μg) were loaded on 10% sodium dodecyl sulfate-polyacrylamide gel and blotted onto nitrocellulose membranes. Blots were incubated with rabbit anti-p27 antibody (Santa Cruz Biotechnology), anti-GAPDH antibody (Chemicon), anti-phospho-Akt antibody and anti-total-Akt antibody (Cell Signaling Technology Inc., Beverly, MA). The relative integrated density of each protein band was digitized by NIH image J 1.34 s.

### 2.4. Evaluation of apoptosis

TUNEL assays were performed using an ApopTag fluorescein in situ apoptosis detection kit (Chemicon International Inc.) according to the manufacturer's instructions as described previously [17]. Nuclear morphology was examined by labeling with DAPI solution (0.6 μg/mL, Dojindo Laboratories). Immunofluorescence staining was performed to confirm α-smooth muscle actin (αSMA) expression using αSMA antibody (1:100 dilution, Sigma). Caspase assay was performed using a CaspaTag Caspase-3/7 in situ apoptosis detection kit (Chemicon International Inc.) according to the manufacturer's instructions. Nuclear morphology was examined by Hoechst staining. The samples were analyzed by fluorescence microscopy (Olympus IX71, Olympus Optical Co. Ltd, Tokyo, Japan). For each cover slip, 5–10 fields (with 10–30 cells in each field) were randomly selected to determine the percentage of apoptotic cells in total cells based on the morphological characteristics of apoptosis. PASMCs were reseeded on collagen-coated glass cover slips in 12-well plates at a density of $5\times10^4$ cells/well on day 0. After 16 h of incubation (on day 1), the culture media were replaced with low-serum culture media (DMEM, 0.1% FBS, and 0.1 mg/mL kanamycin), and the cultured cells were made quiescent for 48 h. On day 3, PDGF-BB (10 ng/mL), imatinib (1 μg/mL) or Akt-I-1/2 (1 μmol/L) was added to the media. After 24 h (on day 4), the cells were stained by using an ApopTag fluorescein in situ apoptosis detection kit or CaspaTag in situ apoptosis detection kit.

Transmission electron microscopy was performed with an electron microscope (H-7100: Hitachi; Tokyo, Japan).

To observe cellular apoptosis with a time-lapse system (Olympus Optical Co.), PASMCs were cultured on a 35-mm culture dish that has a micro-photolithographed squared pattern (Kuraray Co., Ltd., Tsukuba, Japan) [7] so that the apoptotic cells will not disappear from view.

### 2.5. Statistical analysis

All results are expressed as mean ± SE. Statistical significance for comparison between the two measurements was determined using Student's *t* test. For comparison between the different treatment groups, statistical analysis was performed using one-

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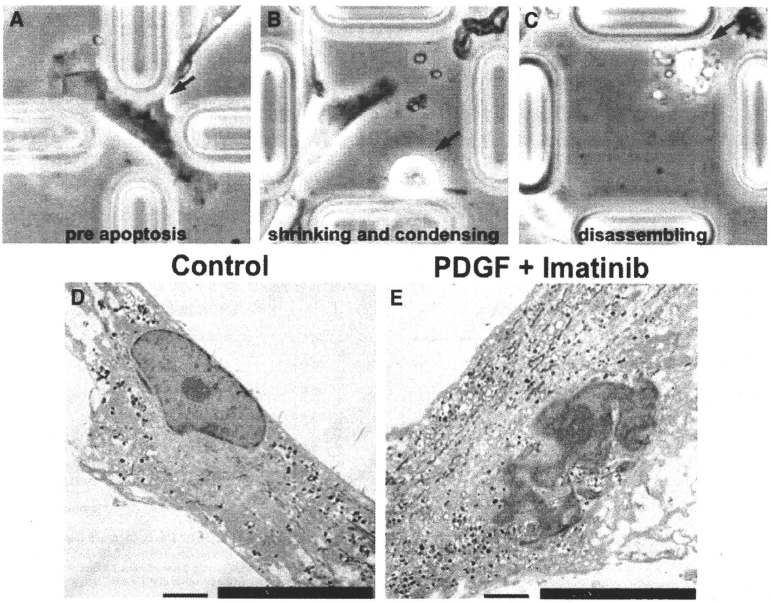


**Fig. 3.** Effect of imatinib on apoptosis of PASMCs in time-lapse microscopy and transmission electron microscopy. A to C, Representative images of time-lapse microscopy. IPAH-PASMCs were treated with imatinib (1 μg/mL) and PDGF-BB (10 ng/mL). Bar = 20 μm. D and E, Representative images of transmission electron microscopy. D, IPAH-PASMCs without treatment (control). E, IPAH-PASMCs treated with imatinib (1 μg/mL) and PDGF-BB (10 ng/mL). Bar = 5 μm.

way ANOVA with Fisher's PLSD test. Values of $P<0.05$ were considered to be statistically significant.

## 3. Results

### 3.1. Inhibitory effect of imatinib on proliferation of PASMCs from IPAH patients

Treatment with imatinib inhibited PDGF-BB-induced proliferation of PASMCs from IPAH patients as assessed by $^{3}$H-thymidine incorporation (n = 5–12 experiments in each cell) (Fig. 1). This result is consistent with recent findings of other investigators [10].

### 3.2. Effect of imatinib on apoptosis of PASMCs from IPAH patients

We performed a TUNEL assay using an ApopTag fluorescein to assess the effect of imatinib on apoptosis of PASMCs from IPAH patients. Fig. 2 shows representative cases of the TUNEL assay. TUNEL-positive cell (green) was observed after 24-hour treatment with imatinib (1 μg/mL) in the presence of PDGF-BB (10 ng/mL) (Fig. 2D and H). However, imatinib (1 μg/mL) (Fig. 2B and F) or PDGF-BB (10 ng/mL) (Fig. 2C and G) alone did not induce apoptosis in IPAH-PASMCs. Imatinib (1 μg/mL) in the presence of PDGF-BB (10 ng/mL) significantly increased TUNEL-positive cells in IPAH-PASMCs compared with the control condition in IPAH-PASMCs ($P<0.05$: 15.1 ± 5.4% versus 4.5 ± 1.3%, n = 4 or 5 experiments in each cell line) (Fig. 2J). There was no significant difference in the percentage of TUNEL-positive cells between the imatinib alone or PDGF alone condition and the control condition (Fig. 2J). There was also no significant difference between the imatinib alone, PDGF alone or both imatinib and PDGF condition and the control condition in normal PASMCs (P = NS, n = 5 experiments) (Fig. 2I).

Fig. 3A, B and C shows the apoptosis induced by the combination of imatinib (1 μg/mL) and PDGF-BB (10 ng/mL) in IPAH-PASMCs as assessed by time-lapse microscopy. A PASMC shows shrinking and condensing and finally disassembling. Fig. 3E shows a transmission electron microscopic image of an apoptotic cell in IPAH-PASMCs. Condensation of chromatin along the nuclear membrane and fragmentation of the nucleus were observed in cultured IPAH-PASMCs treated with imatinib (1 μg/mL) and PDGF-BB (10 ng/mL).

Fig. 4 shows representative cases of the caspase assay in IPAH-PASMCs. Caspase-3 and −7-active cell was observed after 24-hour treatment with imatinib (1 μg/mL) in the presence of PDGF-BB (10 ng/mL) (Fig. 4D and H). Imatinib (1 μg/mL) in the presence of PDGF-BB (10 ng/mL) significantly increased caspase-3 and −7-active cells in IPAH-PASMCs compared with the control condition ($P<0.01$: 12.4 ± 3.0% versus 2.2 ± 1.2%, n = 5 experiments in each cell line) (Fig. 4J). There was no significant difference in the percentage of caspase-3 and −7-positive cells between the imatinib alone or PDGF alone condition and the control condition in IPAH-PASMCs (Fig. 4J). There was also no significant difference between the imatinib alone, PDGF alone or both imatinib and PDGF condition and the control condition in normal PASMCs (P = NS, n = 5 experiments) (Fig. 3I).

These results show that imatinib did not induce apoptosis in normal PASMCs and quiescent IPAH-PASMCs but that imatinib had a proapoptotic effect on IPAH-PASMCs stimulated with PDGF.

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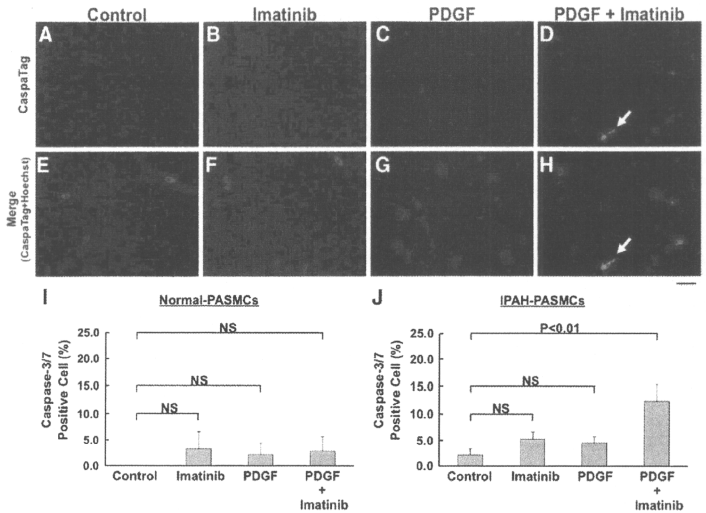


**Fig. 4.** Effect of imatinib on apoptosis of PASMCs in Caspase assay using a CaspaTag Caspase-3/7 in situ apoptosis detection kit. A to D, CaspaTag staining (green). E to H, Combined images (merge) of CaspaTag staining and Hoechst nuclear staining (blue). A and E, IPAH-PASMCs without treatment. B and F, IPAH-PASMCs treated with imatinib (1 μg/mL). C and G, IPAH-PASMCs treated with PDGF-BB (10 ng/mL). D and H, IPAH-PASMCs treated with imatinib and PDGF-BB. Arrow shows a caspase-3/7-positive cell (green). Bar = 500 μm. I, Effect of imatinib on apoptosis of normal PASMCs in Caspase assay. J, Effect of imatinib on apoptosis of IPAH-PASMCs in Caspase assay. Imatinib (1 μg/mL) in the presence of PDGF-BB (10 ng/mL) significantly increased caspase-positive (apoptotic) cells in IPAH-PASMCs compared with the control condition ($P<0.01$). Data are mean ± SE.

### 3.3. *Effect of imatinib on PDGF-BB-induced phosphorylation of Akt*

Western blot analysis revealed that PDGF-BB induced phosphorylation of Akt at 15 min (Fig. 5A, lanes 2 and B). Akt phosphorylation was significantly inhibited by imatinib (1 ng/mL) compared with the treatment with PDGF-BB ($P<0.05$, $n=4$ experiments) (Fig. 5A, lanes 3 and B).

Akt-I-1/2 (1 μmol/L), an Akt inhibitor, could mimic the effects of imatinib on PASMCs. Akt-I-1/2 significantly inhibited PDGF-induced proliferation of IPAH-PASMCs as assessed by $^3$H-thymidine incorporation ($P<0.001$, $n=10$ experiments) (Fig. 5C). Akt-I-1/2 in the presence of PDGF-BB significantly increased TUNEL-positive cells ($P<0.05$, $n=5$ experiments) (Fig. 5D) and caspase-3,7-positive cells in IPAH-PASMCs ($P<0.05$, $n=5$ experiments) (Fig. 5E) compared with the control condition. These results show that the inhibition of Akt is strongly related to the anti-proliferative and pro-apoptotic effects of imatinib on PDGF-stimulated IPAH-PASMCs.

## 4. Discussion

Two major new findings were obtained in the present study. First, imatinib did not induce apoptosis in quiescent IPAH-PASMCs and normal PASMCs, but it had a pro-apoptotic effect on IPAH-PASMCs stimulated with PDGF. Second, inhibition of Akt is related to the anti-proliferative and pro-apoptotic effects of imatinib on PDGF-stimulated IPAH-PASMCs.

Imatinib alone did not induce apoptosis in IPAH-PASMCs. This result is consistent with recent findings of other investigators [10]. However, the combination of imatinib and PDGF induced apoptosis. Therefore, imatinib did not induce apoptosis in quiescent IPAH-PASMCs, but it had a pro-apoptotic effect on IPAH-PASMCs stimulated with PDGF. It has been reported that PDGF-A and PDGF-B mRNA levels were increased in small pulmonary arteries from patients with IPAH [10] and that serum PDGF-BB levels across the lung circulation were higher in IPAH patients [18]. Therefore, imatinib is expected to induce apoptosis in clinical settings. Further studies are needed to clarify this point.

Many signaling pathways, including ERK, p38 MAPK and Akt, are involved in proliferation and survival of PASMCs [14,19]. Akt is a member of the serine/threonine-specific kinase family known for facilitating cell survival via the inhibition of apoptotic pathways. It has been shown that PDGF stimulation transiently phosphorylates Akt and the mammalian target of rapamycin (mTOR) in PASMCs from patients with chronic thromboembolic pulmonary hypertension [19]. In our study, PDGF-BB induced phosphorylation of Akt and it was inhibited by imatinib in IPAH-PASMCs. Akt-I-1/2, an Akt inhibitor, could mimic the effects of imatinib on PASMCs. Akt is related to the anti-proliferative and pro-apoptotic effects of imatinib on PDGF-stimulated IPAH-PASMCs.

Imatinib is a drug used for treating chronic myelogenous leukemia and gastrointestinal stromal tumors. However, resistance to imatinib can occur [20–22]. Not only primary resistance within the first two months but also secondary resistance develops after a median of about 2 years of treatment with the drug. Hatano et al. reported that imatinib decreases the plasma concentration of PDGF-BB in patients with PAH, while the improvement in hemodynamic parameters is transient [11]. We showed that imatinib had a pro-apoptotic effect on IPAH-PASMCs stimulated with PDGF in the present study. Thus, imatinib would induce apoptosis only in the early period of treatment when plasma PDGF-BB levels are relatively high. After the PDGF levels have decreased, imatinib would not be able to induce apoptosis. Therefore, resistance to imatinib might occur in patients with pulmonary hypertension. Attention is needed in clinical use.

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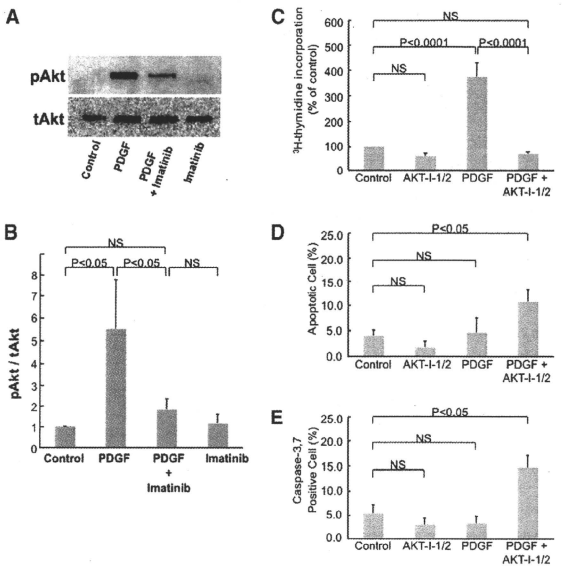


**Fig. 5.** Effect of imatinib on PDGF-BB-induced phosphorylation of Akt and effects of an Akt inhibitor on PDGF-BB-stimulated proliferation and apoptosis of PASMCs. A, Western blot analysis of total Akt (tAkt) and phosphorylated Akt (pAkt). PDGF-BB (10 ng/mL) induced phosphorylation of Akt at 15 min (lanes 2). Akt phosphorylation was inhibited by imatinib (1 ng/mL) (lanes 3). B, Bar graphs show semiquantitive analysis of pAkt expression level in IPAH-PASMCs. Data are mean ± SE of the intensity of the band corresponding to pAkt relative to tAkt. C, Anti-proliferative effect of Akt-I-1/2 (1 μmol/L), an Akt inhibitor, on IPAH-PASMCs stimulated with PDGF-BB (10 ng/mL). $^3$H-thymidine incorporation was measured. Counts per minute (cpm) were expressed as a percentage of cpm of IPAH-PASMCs treated with a diluent (control). Data are mean ± SE. D, Effect of Akt-I-1/2 (1 μmol/L) on apoptosis of PASMCs in TUNEL assay by ApopTag fluorescein. E, Effect of Akt-I-1/2 (1 μmol/L) on apoptosis of PASMCs in Caspase assay using a CaspaTag Caspase-3/7 in situ apoptosis detection kit.

In conclusion, imatinib inhibited PDGF-induced proliferation of IPAH-PASMCs. Imatinib did not induce apoptosis in quiescent IPAH-PASMCs, but it had a pro-apoptotic effect on IPAH-PASMCs stimulated with PDGF. Inhibition of Akt may be important in the anti-proliferative and pro-apoptotic effects of imatinib on PDGF-stimulated IPAH-PASMCs. Modulation of PDGF signaling such as Akt is important. Inhibition of PDGF signaling by imatinib may become a useful molecular-targeted therapy for IPAH.

## Conflict of interest

There are no relationships with industry.

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## Prednisolone Ameliorates Idiopathic Pulmonary Arterial Hypertension

*To the Editor:*

Idiopathic pulmonary arterial hypertension (IPAH) is caused by pulmonary vascular remodeling. The entire mechanism by which pulmonary vascular remodeling develops has not been well elucidated. Inflammation or autoimmune background are implied in many cases (1, 2). However, there are no proven therapies that modulate inflammatory processes to treat IPAH. We have previously reported that prednisolone has an inhibitory effect on the proliferation and migration of pulmonary arterial smooth muscle cells (PASMC) from patients with IPAH (3). Here, we report a case of IPAH coexisting with idiopathic thrombocytopenic purpura (ITP), which was successfully treated with prednisolone. The severity of IPAH was ameliorated by prednisolone therapy.

A 34-year-old female, who was diagnosed with IPAH three years earlier, was admitted to our hospital for further evaluation and treatment of thrombocytopenia. After detailed examination by a hematologist, she was diagnosed with ITP. Bone marrow aspiration showed a normal number of megakaryocytes, and platelet-associated IgG was elevated to 135.4 ng/$10^7$ cells (normal range: 9.0–25.0 ng/$10^7$ cells). A rheumatologist excluded definite diagnosis of any collagen diseases. One mg/kg/day (65 mg/body/d) of oral prednisolone was started as a standard therapy for treatment of ITP (Figure 1). During prednisolone therapy, the dose of epoprostenol (94 ng/kg/min) and diuretics (trasemide, 8 mg/d) were maintained at the same levels. Her condition progressively had improved and she gradually lost 3.3 kg in body weight. She felt less dyspnea, and World Health Organization (WHO) functional class improved from class III to II. Six-minute-walk distance increased to 305 m, although she could not walk for 6 minutes before steroid therapy because of shortness of breath. The cardiothoracic ratio decreased from 64% to 56%. Pericardial effusion decreased from 17 to 4 mm and the Doppler-estimated peak systolic tricuspid regurgitation pressure gradient decreased from 75 to 50 mm Hg. No adverse effects were observed during prednisolone treatment. While tapering prednisolone, she started to feel slight increase in shortness of breath upon exertion. Platelet counts were successfully maintained within normal range after termination of steroid therapy. However, the tricuspid regurgitation pressure gradient again increased, and we resumed increasing the dose of epoprostenol. Although she was discharged from our hospital at that time, she underwent living-donor lobar lung transplantation due to progressive heart failure 5 months later.

It is well documented that inflammation is involved in the development of pulmonary vascular remodeling and the pathogenesis of IPAH. Elevated serum levels of interleukin-1β and -6 and circulating monocyte chemoattractant protein-1 are reported in patients (2, 4). T- and B-lymphocytes and macrophages are found to accumulate in plexiform lesions (1, 5). Prednisolone is a drug that has anti-inflammatory, immunosuppressive, and anti-proliferative effects (6, 7). The effectiveness of steroid therapy in pulmonary hypertension associated with collagen diseases is already reported (8–10). However, there is no report that demonstrates the effectiveness of prednisolone therapy in IPAH alone, without any association with collagen diseases.

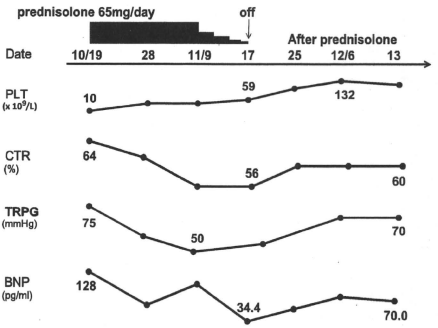


*Figure 1.* Time course of laboratory data and pulmonary hypertension before and after prednisolone treatment. Platelet counts (PLT), cardiothoracic ratio (CTR), Doppler-estimated peak systolic tricuspid regurgitation pressure gradient (TRPG), and plasma level of brain natriuretic peptide (BNP) are shown.

We have previously shown that prednisolone significantly inhibited platelet-derived growth factor (PDGF)-induced accelerated proliferation and migration of PASMC from patients with IPAH (3). Moreover, we demonstrated that the effect was caused, at least in part, by inhibiting activation of NF-κB, a key transcription factor that controls immunity, inflammation, cell proliferation, and apoptosis (11).

In summary, this case describes the potential effectiveness of prednisolone for the treatment of IPAH. The rationale for such treatment derives from its potent antiproliferative effect on PASMC from patients with IPAH demonstrated *in vitro*. We recognize that it is still difficult to integrate these results to apply prednisolone to all patients, taking the many side effects of prednisolone into account. However, this case supports the notion that prednisolone can inhibit pulmonary vascular remodeling and could be considered as a new targeted therapy for IPAH.

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## An Unusual Localized Progressive Fibrotic Cavity Mimicking Lung Malignancy in Idiopathic Pulmonary Fibrosis

*To The Editor:*

Idiopathic pulmonary fibrosis (IPF) is defined as a chronic fibrosing interstitial pneumonia of unknown cause limited to the lungs (1). Because of the increased risk in developing lung cancer, physicians must make their best efforts in their differential diagnoses of pulmonary cavities in patients with IPF. In this report, we describe a cavitary lesion resembling a cavitary mass in IPF histopathologically diagnosed as localized progressive fibrosis.

A 72-year-old man was admitted for further evaluation of a cavitary lung mass. Three years previously, the patient had been diagnosed as having IPF (Figure 1A) and treated with oral prednisolone and cyclophosphamide. On high-resolution computed tomography (HRCT) at 4 months before admission, a cavitary nodule 2.3 × 1.5 cm in size and 5 mm in wall thickness in the left upper lobe had been initially detected (Figure 1B). HRCT on admission showed a cavity containing a mural nodule 3.7 × 1.9 cm in size and 13 mm in wall thickness (Figure 1C). Contrast-enhanced CT of chest revealed an enhancement of the cavitary wall with suspicion of an aspergilloma or pulmonary tuberculosis. However, there was no evidence of infection of bacteria or fungus in sputum and bronchial washing fluids. Percutaneous transthoracic needle biopsy was performed. Histopathologic examination disclosed chronic inflammation with dense fibrosis, focal organization, and infiltration of neutrophils. On follow-up HRCT at 10 months after discharge, the cavity regrew to 2.7 × 2.6 cm (Figure 1D). The wall thickness of the cavity was increased to 18 mm, with a volume decrease in the involved pulmonary lobe. Moreover, diffuse ground glass opacity with diffuse honeycombing predominantly in the subpleural location was aggravated as compared with previous scans. Fluoroscopically guided percutaneous lung biopsy was performed again, but there was no evidence for infection or malignancy. A wedge resection was performed for the cavitary mass. Pathologic examination showed nonspecific chronic inflammation with dense fibrosis, and negative results of fungal and mycobacterial stains (Figures 1E and 1F). There was no evidence for recurrence through 20 months after the resection.

This is an interesting case of a pulmonary cavitary lesion histopathologically diagnosed as progressive fibrosis by repetitive biopsies, which could be misdiagnosed as aspergilloma or lung malignancy on serial HRCT. IPF is associated with an increased risk of various pulmonary diseases such as lung cancer, pulmonary tuberculosis, aspergillosis, and other respiratory infectious diseases. In addition, our case supports the existence of a rare entity, the localized progressive fibrosis presenting as cavitary lesions in IPF, although the prevalence is very low. Therefore, a pulmonary cavity with preexisting IPF should always carefully be evaluated and confirmed even though an invasive diagnostic modality may be used. The localized progressive fibrosis in our patient suggests that pulmonologists may include it in their differential diagnoses of cavitary lung lesions developed in IPF.

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## Is the Reference Arterial pH Higher than Usually Acknowledged?

*To the Editor:*

Since the invention of the blood gas apparatus by Severinghaus and Bradley in 1959 (1), arterial blood gas analysis has become

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# Electroanatomical Correlation of Repolarization Abnormalities in Brugada Syndrome

## Detection of Type 1 Electrocardiogram in the Right Ventricular Outflow Tract

**To the Editor:** ST-segment elevation is the most important characteristic for the diagnosis of Brugada syndrome (BrS). The Consensus Report of Brugada Syndrome defined type 1 electrocardiogram (ECG) (coved-type ST-segment elevation ≥0.2 mV with a negative T-wave in the right precordial lead) is diagnostic for BrS (1,2). Although the standard ECG recording of the right precordial leads at the fourth intercostal space (ICS) sometimes fails to reveal type 1 ECG, additional recording of leads $V_1$ and $V_2$ at high (third and second) ICS increases the sensitivity for detecting type 1 ECG (3,4).

ST-segment elevation in BrS is believed to represent abnormal repolarization at the right ventricular outflow tract (RVOT) (3). However, the relationship between ECG recording site (standard and high ICS recording) and the anatomical position of the RVOT is still unclear. Accordingly, we examined the relationship between the lead positions of type 1 ECG and the location of the RVOT in patients with BrS.

We examined 60 patients with BrS (59 men; 47 ± 12 years of age). We defined BrS based on the Second Consensus Report of Brugada Syndrome (2). Cardiac catheterization, electrophysiologic study, and genetic analysis were performed according to the protocol approved by the ethics committee of Okayama University. Written informed consent was obtained from all patients. Cardiac catheterization and ECG recording were performed simultaneously in all patients. The ECG was recorded at the additional third and standard fourth ICS in leads $V_1$ and $V_2$ with fluoroscopically visible electrodes. Anatomical location of the RVOT was determined under fluoroscopic images, with right ventriculography performed in the right anterior oblique (RAO) view. We determined the location of the RVOT as being below the pulmonary valve and above the anterior border of the tricuspid valve in the end-diastole and -expiration phase. We examined the relationships between the location of the RVOT and the position of leads manifesting type 1 ECG. For detailed analysis of lead position, we made an 8-segment model of the right ventricle in RAO view. We analyzed the lead position and appearance of type 1 ECG at baseline conditions. If patients did not have type 1 ECG in any leads at baseline, we used an intravenous injection of a pure sodium-channel blocker, pilsicainide (n = 39). Genetic screening revealed that SCN5A mutation was present in 5 of 39 patients (12.8%).

Anatomic correlation of the RVOT and the locations of the third and fourth ICS were variable in each patient (Figs. 1A and 1B). Figure 1A shows an example of 1 patient in whom the RVOT was located at the fourth ICS and type 1 ECG was recorded in leads $V_1$ and $V_2$ at both the third and fourth ICS. Figure 1B shows an example of another patient in whom the RVOT corresponded to the third ICS, and the fourth ICS coincided with inflow to the anterior free wall of the right ventricle. Type 1 ECG was recorded only by the third ICS electrode in this patient. Overall, type 1 ECG was recorded in leads that corresponded with the RVOT.

Figure 1C shows the distribution of location of ECG leads with and without type 1 ECG. The location of the RVOT corresponded to the $V_1$ and $V_2$ leads at the fourth ICS in 11 patients (18.3%) and at the third ICS in 49 patients (81.7%). In 9 of 11 patients (82%) in whom the fourth ICS represented the RVOT, type 1 ECG was obtained at the fourth ICS. However, 41 of 49 patients (84%) in whom the third ICS represented the RVOT did not show type 1 ECG at the fourth ICS, but it was shown at the third ICS instead. Accordingly, most of the ECG leads manifesting type 1 were distributed at the RVOT ($p < 0.0001$). Documented VF (n = 12), syncope (n = 10), family history of sudden cardiac death (n = 19), induced ventricular fibrillation with programmed electrical stimulation (n = 30), presence of SCN5A mutation, and spontaneous type 1 ECG (n = 21) were not related to the location of the ECG leads corresponding with the RVOT.

In the present study, we showed that the RVOT determines the manifestation of type 1 ECG in patients with BrS. And the relationship between the position of the leads and the RVOT was variable in individual cases. Abnormal repolarization is thought to be associated with the RVOT in BrS. However, the exact anatomical location of the formation of type 1 ECG has not been fully examined yet. Because the $V_1$ and $V_2$ leads at the standard fourth ICS were not associated with the RVOT in approximately 80% of patients with BrS, the recording of additional $V_1$ and $V_2$ leads at the third ICS improved the detection of type 1 ECG in BrS.

Previous basic studies have shown that the transient outward current (Ito) is most prominent in the RVOT region of the ventricular myocardium, giving rise to a more prominent action potential notch in this region, which is responsible for inscription of the accentuated J-wave or ST-segment elevation in this lead facing this region of the right ventricle (5).

In summary, our results show that positioning of ECG electrodes at the RVOT can detect type 1 ECG in patients with BrS. The ICS, which is associated with the RVOT, can be different in each patient.

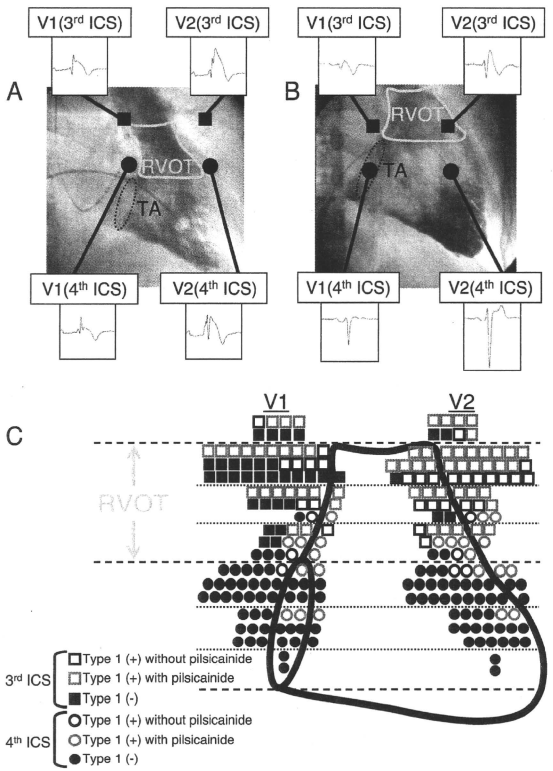


**Figure 1** **Detection of Type 1 ECG in the RVOT**

**(A)** A 62-year-old male patient with recurrent unknown syncope attacks. **(B)** A 17-year-old male patient without any symptoms. **(C)** Position of electrocardiogram (ECG) leads in all patients. The lead location in front of the right ventricular outflow tract (RVOT) determined the manifestation of type 1 ECG. The lead position manifesting type 1 ECG coincided with the location of the RVOT in 34 of 44 (77.3%) $V_1$ leads and in 58 of 71 (81.7%) $V_2$ leads. ICS = intercostal space; TA = tricuspid annulus.

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## Letters to the Editor

# Exercise-Induced Troponin Elevation

## Not Necessarily a Benign Phenomenon

In their comprehensive review of exercise-induced cardiac troponin elevation, Shave et al. (1) come to the conclusion that this is a benign phenomena most likely related to leakage of troponin from the cardiac myocyte membrane rather than myocyte necrosis—the usual cause of troponin elevation. In the absence of long-term follow-up of elite ultra-endurance athletes, this conclusion should be viewed with caution. There is increasing evidence that, in some athletes, participation in multiple extreme endurance events over a long period of years can lead to abnormal right ventricular (RV) enlargement, dysfunction, and—more ominously—potentially lethal arrhythmias (2–4). Such athletes are clinically and genetically distinct from those suffering from familial arrhythmogenic RV dysplasia/cardiomyopathy (5). An appropriate terminology for such individuals is "exercise-induced right ventricular dysplasia" (2–5). A plausible hypothesis for this syndrome is that extreme endurance exercise places a strain on the RV that on occasions leads to myocardial necrosis, albeit small, as reflected in post-exercise elevated troponin levels. The cumulative effect of repeated episodes of necrosis can eventually lead to sufficient fibrosis to result in RV dysfunction and to act as a substrate for potentially lethal arrhythmias. How prevalent exercise-induced RV dysplasia is and whether there is a genetic predisposition to the condition remains to be determined.

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### Reply

We appreciate Dr. Harper's interest in our recent report (1). Dr. Harper suggests that without reassuring evidence from longitudinal studies it is possible that ultra-endurance exercise can produce a variant of right ventricular cardiomyopathy and suggests that the elevated cardiac troponin (cTn) observed after exercise are not benign. From a historical viewpoint, ultra-endurance events are not new. At the turn of the last century walking and running contests of several days duration were popular spectator events and raised concerns similar to those of Dr. Harper, but concerns about bicyclists', runners', and rowers' hearts were never documented (2). Right ventricular enlargement from endurance activity is not unusual but is rather one of the expected adaptations from exercise training. Indeed, all 4 cardiac chambers enlarge with exercise training, but there is little evidence of right ventricular dysfunction, except for reversible, transient changes after endurance events (3)—which might be related to a number of factors, such as elevations in heart rate, plasma volume alterations, or desensitization of beta-adrenoceptors. Furthermore, the observations that cTn is elevated after low-intensity exercise such as walking (4), early during a treadmill marathon (5), and after 30 min of high-intensity running (6) suggest that cTn elevations are common with exercise and not necessarily a product of prolonged effort. Recent studies have also shown no relationship between exercise-induced cTn release and late gadolinium enhancement with cardiac magnetic resonance imaging (7) and that, unlike acute coronary syndromes, cTn rapidly returns to baseline after exercise. In combination, such observations suggest a benign event. Finally, we would caution Dr. Harper as he has us: in the absence of longitudinal studies, it is provocative and premature to suggest that right ventricular problems observed in a case series of endurance athletes are produced by their athletic participation.

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ORIGINAL ARTICLE
Arrhythmia/Electrophysiology

# *SCN5A* Mutation Is Associated With Early and Frequent Recurrence of Ventricular Fibrillation in Patients With Brugada Syndrome

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*Background:* Mutations in *SCN5A* are reportedly linked to Brugada syndrome (BS), but recent observations suggest that they are not necessarily associated with ventricular fibrillation (VF) in BS patients. Therefore, the clinical importance of *SCN5A* mutations in BS patients was examined in the present study.

*Methods and Results:* The 108 BS patients were examined for *SCN5A* mutations and various parameters were compared between patients with and without mutations. An implantable cardioverter defibrillator (ICD) was implanted in 49 patients and a predictor of appropriate ICD shock was investigated. The existence of a *SCN5A* mutation was not associated with initial VF episodes (21.7% vs 20.0%, P=0.373). In the secondary prevention group, appropriate shock-free survival rate was significantly lower in patients with spontaneous type 1 ECG than in those without (41.1% vs 85.7% at 2 years, P=0.014). The appropriate shock-free survival rate was also significantly lower in patients with *SCN5A* mutations than in those without (28.6% vs 83.3% at 1 year, P=0.040). Appropriate shock was more frequent in patients with *SCN5A* mutations than in those without (6.6±6.2 vs 1.7±3.0, P=0.007).

*Conclusions:* *SCN5A* mutations are associated with early and frequent VF recurrence, but not with initial VF episodes. This is the first report on the genotype–phenotype interaction and clinical significance of this mutation. (*Circ J* 2010; **74:** 2572–2578)

Key Words: Appropriate ICD shock; Brugada syndrome; Genotype–phenotype interaction; Implantable cardioverter defibrillator; *SCN5A* mutation

Brugada syndrome (BS) is characterized by ST-segment elevation in the right precordial leads and sudden death (SD) because of ventricular fibrillation (VF).[1,2] *SCN5A* encodes for the alpha subunit of the cardiac sodium channel gene and BS is associated with *SCN5A* mutations in approximately 15% of probands.[3–5]

Previous studies have suggested that *SCN5A* mutations are associated with depolarization abnormalities such as bradyarrhythmia, conduction delay and conduction block.[6–14]

However, it is unclear whether BS patients with *SCN5A* mutations have a greater risk of arrhythmic events or SD. Recent observations have also revealed that *SCN5A* mutations are not necessarily associated with VF or syncopal episodes in patients with BS.[15,16]

Thus, the clinical importance of *SCN5A* mutation for developing VF in BS patients is not clear.

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## Methods

### Patients

The subjects were 108 consecutive BS patients who were admitted to Okayama University Hospital, Fukuoka University Hospital, Cardiovascular Center Sakakibara Hospital, National Hospital Organization Okayama Medical Center and Fukuyama Cardiovascular Hospital during January 1997 to December 2009. All 108 BS patients were examined for *SCN5A* mutations and we divided them into 2 groups according to the presence or absence of a mutation. All patients underwent echocardiography and chest X-ray and no abnormalities were found. All of the tests that were performed were approved by the medical ethical review committees of each hospital and informed consent was given by all patients.

### Clinical Examination

All BS patients had a type 1 ECG recorded spontaneously and after provocation with a class I antiarrhythmic drug[3] (pilsicainide 1 mg/kg body weight at 10 mg/min IV).[17] We evaluated the ECG before drug administration, immediately after, and at 5 and 10 min after drug administration.

An electrophysiological study was performed in 99 of the 108 patients. Induction of ventricular arrhythmia (VA) was attempted without using any antiarrhythmic drugs. The criterion for induction of VA was induction of sustained polymorphic ventricular tachycardia (VT) or VF by programmed electrical stimulation (PES) from the right ventricular apex or right ventricular outflow tract. The protocol of ventricular stimuli included up to 3 extrastimuli (2 basic cycle lengths of 600 and 400 ms) and rapid ventricular pacing, with the coupling interval of the extrastimuli not being less than 180 ms and the ventricular rate of rapid burst pacing not exceeding 270 beats/min. The electrophysiological study was performed as reported previously.[18] We also measured the His–ventricular (HV) interval.

The ECGs were measured by an investigator who was unaware of patient characteristics. QRS duration was analyzed in lead $V_5$ from the standard 12-lead ECG in all BS patients.

Signal-averaged electrocardiograms (SAECG) (ART 1200EPX; noise level <0.3 $\mu$V, high-pass filtering of 40 Hz using a bidirectional 4-pole Butterworth) were obtained in all BS patients. Filtered QRS duration, root mean square voltage of the terminal 40 ms in the filtered QRS complex (RMS40) and duration of low-amplitude signals <40 $\mu$V in the terminal filtered QRS complex (LAS40) were measured by SAECG. A late potential (LP) was considered to be positive when the 2 criteria (RMS40<20 $\mu$V and LAS40>40 ms) were met.[19]

In the absence of symptoms or device therapy (implantable cardioverter defibrillator: ICD), all BS patients were seen routinely every 3–12 months for clinical review and device interrogation, according to local practice. In the event of a shock, patients were seen at the ICD clinic within 48 h and the device was interrogated. Appropriate shocks were defined as shocks delivered for VT or VF.

None of the patients received antiarrhythmic drugs at the time of first admission. We started treatment with antiarrhythmic drugs in patients with VF if they had experienced recurrent VF episodes and appropriate ICD shocks.

### Mutation Analysis of *SCN5A*

Genetic analysis was performed in compliance with the ethics committee guidelines for human genome studies of the Ethics Committee. Informed consent was given by all 108 subjects. Genomic DNA was extracted from peripheral blood leukocytes using a DNA extraction kit (Gentra, Minneapolis, MN, USA) and was stored at –30°C until use.

In total, 27 exons of *SCN5A* were amplified with previously reported intronic primers.[20] *SCN5A* exon 1 is a noncoding region, and we did not analyze it in this study. Exons 6, 17-1 sense, 21, and 25 were unable to be amplified enough by the primers, and we therefore designed the following intronic primers as previously described.[21–22] The primers used in this study were: exon 6: sense, 5'-GTT ATC CCA GGT AAG ATG CCC-3'; anti-sense, 5'-TGG TGA CAG

**Table 1. Characteristics of the Patients With BS**

| | |
|---|---|
| N | 108 |
| Male, (%) | 105 (97.2) |
| Age (years) | 46.8±11.6 |
| Spontaneous type 1 ECG, (%) | 71 (65.7) |
| Family history of SD, (%) | 30 (27.8) |
| Documented VF, (%) | 23 (21.3) |
| Syncopal episode, (%) | 42 (38.9) |
| VF induced by programmed electrical stimulation, (%) | 48/99 (48.5) |
| Late potential, (%) | 71/105 (67.6) |
| *SCN5A* mutation, (%) | 17 (15.7) |
| ICD implantation, (%) | 49 (45.4) |

BS, Brugada syndrome; SD, sudden death; VF, ventricular fibrillation; ICD, implantable cardioverter defibrillator.

**Table 2. Characteristics of BS Patients With and Without Documented VF**

| | Documented VF (+) | Documented VF (–) | P value |
|---|---|---|---|
| N | 23 | 85 | |
| Male, (%) | 22 (95.7) | 83 (97.6) | 0.666 |
| Age (years), (%) | 45.8±11.5 | 47.0±11.7 | 0.624 |
| Spontaneous type 1 ECG, (%) | 13 (56.5) | 58 (68.2) | 0.294 |
| Family history of SD, (%) | 5 (21.7) | 25 (29.4) | 0.466 |
| VF induced by programmed electrical stimulation, (%) | 13/23 (56.5) | 35/76 (46.1) | 0.175 |
| Late potential, (%) | 16/22 (72.7) | 55/83 (66.3) | 0.565 |
| *SCN5A* mutation, (%) | 5 (21.7) | 17 (20.0) | 0.373 |
| QRS duration (ms) | 107±23 | 97±14 | 0.015 |
| HV interval (ms)* | 45.3±7.4 | 41.3±7.9 | 0.042 |

*No. of patients with and without documented VF who were examined for HV interval was 16 and 78, respectively. HV, His-ventricular. Other abbreviations see in Table 1.

**Table 3. Characteristics of BS Patients With and Without *SCN5A* Mutation**

| | *SCN5A* mutation (+) | *SCN5A* mutation (–) | P value |
|---|---|---|---|
| N | 17 | 91 | |
| Male, (%) | 16 (94.1) | 89 (97.8) | 0.396 |
| Age (years) | 46.4±13.6 | 46.8±11.3 | 0.894 |
| Spontaneous type 1 ECG, (%) | 13 (76.5) | 58 (63.7) | 0.310 |
| Family history of SD, (%) | 3 (17.6) | 27 (29.7) | 0.310 |
| Documented VF, (%) | 5 (29.4) | 18 (19.8) | 0.373 |
| Syncopal episode, (%) | 9 (52.9) | 34 (37.4) | 0.452 |
| VF induced by programmed electrical stimulation, (%) | 4/15 (26.7) | 44/84 (52.4) | 0.066 |
| Late potential, (%) | 13/14 (92.9) | 58/91 (63.7) | 0.030 |
| QRS duration (ms) | 110±13 | 97±17 | 0.004 |
| HV interval (ms)* | 50.1±9.4 | 41.1±7.0 | 0.0001 |

*No. of patients with and without *SCN5A* mutation examined for HV interval was 12 and 82, respectively.
Abbreviations see in Tables 1,2.

**Table 4. Characteristics of BS Patients With ICD Implantation for Secondary or Primary Prevention**

| | Secondary prevention | Primary prevention | P value |
|---|---|---|---|
| N | 31 | 18 | |
| Male, (%) | 29 (93.5) | 18 (100) | 0.271 |
| Age (years) | 47.6±11.5 | 48.7±12.1 | 0.769 |
| Spontaneous type 1 ECG, (%) | 17 (54.8) | 10 (55.6) | 0.961 |
| Family history of SD, (%) | 5 (16.1) | 10 (55.6) | 0.004 |
| VF induced by programmed electrical stimulation, (%) | 18 (58.1) | 14 (77.8) | 0.162 |
| Late potential, (%) | 20/28 (71.4) | 13/18 (72.2) | 0.395 |
| *SCN5A* mutation, (%) | 7 (22.6) | 1 (5.6) | 0.120 |

Abbreviations see in Table 1.

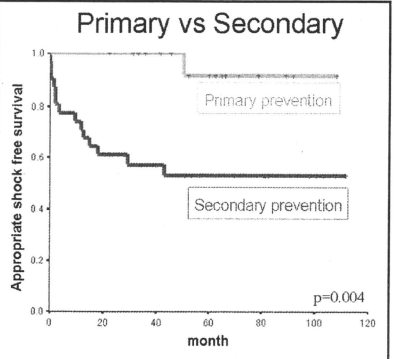


**Figure 1.** Appropriate-shock-free survival rate in Brugada syndrome (BS) patients with an implantable cardioverter defibrillator (ICD) for secondary or primary prevention. 14 (46.7%) of the 31 patients with ICD implantation for secondary prevention (secondary prevention group) and 1 (5.6%) of the 18 patients with ICD implantation for primary prevention (primary prevention group) received appropriate shocks. The appropriate shock-free survival rate was significantly lower in the secondary prevention group than in the primary prevention group (52.8% vs 91.7% at 5 years, P=0.004).

GCA CAT TCG AAG-3'; exon 17-1: sense, 5'-AAG CCT CGG AGC TGT TTG TCA CA-3'; exon 21: sense, 5'-TGC CTG GTG CAG GGT GGA AT-3'; anti-sense, 5'-ACT CAG ACT TAC GTC CTC CTT C-3'; exon 25: sense, 5'-TCT TTC CCA CAG AAT GGA CAC C-3'; anti-sense, 5'-AAG GTG AGA TGG GAC CTG GAG-3'. Polymerase chain reaction (PCR) was performed in a 20-$\mu$l reaction volume containing 50 ng of genomic DNA, 20 pmol of each primer, 0.8 mmol/L dNTPs, 1×reaction buffer, 1.5 mmol/L MgCl2, and 0.7 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) or TAKARA Taq (Takara Bio Inc, Otsu, Japan). All PCR products were treated with exonuclease I (New England BioLabs, Ipswich, MA, USA) and shrimp alkaline phosphatase (USB Corporation, Cleveland, OH, USA), reacted with a Big Dye Terminator v1.1 cycle sequencing kit (Applied Biosystems), and analyzed on an ABI PRISM3130xl sequencer (Applied Biosystems). The mutations were confirmed 4 times by independent PCR amplification and sequencing.

### Statistical Analysis

Data are expressed as mean values±standard deviation. Student's t-test was performed to test for statistical differences between 2 unpaired mean values, and categorical data and percentage frequencies were analyzed by the chi-square test. The event-rate curve was generated according to the Kaplan-Meier method (SPSS II for Windows, SPSS Inc, Chicago, IL, USA). A P value <0.05 was considered significant.

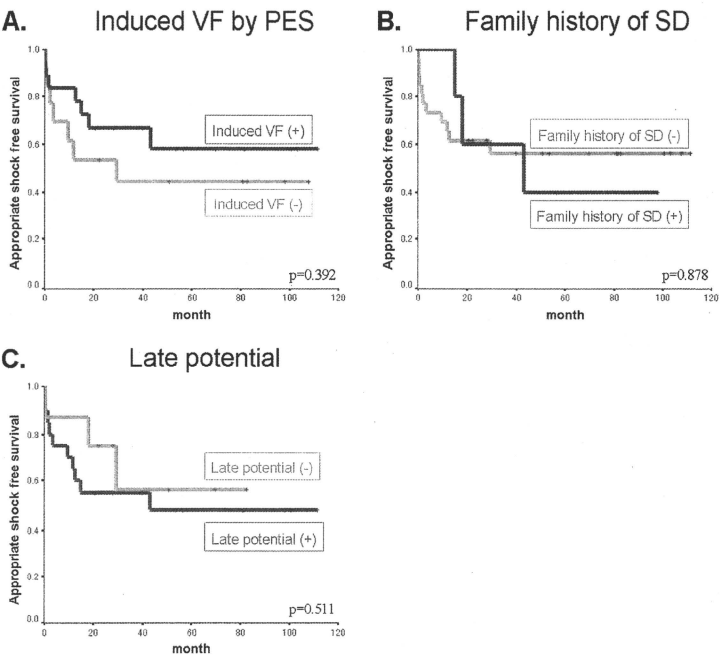


Figure 2. Appropriate-shock-free survival rate in Brugada syndrome (BS) patients with an implantable cardioverter defibrillator (ICD) for secondary prevention. There was no significant difference in the appropriate-shock-free survival rate between BS patients with and without (A) ventricular fibrillation (VF) induced by programmed electrical stimulation (PES), (B) family history of sudden death (SD) and (C) late potentials.

## Results

### Clinical Difference Between BS Patients With and Without Documented VF

Baseline characteristics of the patients are shown in Table 1. There were no significant differences in sex, age, family history of SD, VF induced by PES, LPs, spontaneous type 1 ECG and *SCN5A* mutation between patients with or without documented VF; however, QRS duration and HV interval were significantly longer in patients with documented VF (Table 2).

### Clinical Difference Between BS Patients With and Without *SCN5A* Mutation

As shown in Table 3, there was no significant difference in documented VF between patients with or without a *SCN5A* mutation, nor were there significant differences in sex, age, family history of SD, syncopal episodes, VF induced by PES and spontaneous type 1 ECG between the 2 groups. However, patients with *SCN5A* mutations were significantly more likely to have LPs than were those without a mutation. QRS duration and HV interval were also significantly longer in patients with a *SCN5A* mutation.

### Clinical Course of BS Patients With ICD

An ICD was implanted in 49 of the 108 patients, for secondary prevention in 31 patients (secondary prevention group, which included 14 patients with syncope only, and 17 patients with documented VF at the time of ICD implantation) and for primary prevention in 18 patients (primary prevention group) (Table 4). During a mean follow-up period of 71.9±41.3 months after ICD implantation, no cardiac deaths occurred. Of the 49 patients 15 (30.6%) had appropriate ICD shocks. All cases of spontaneous VF were successfully terminated by only 1 ICD shock. The patients with frequent VF occurrence were treated with drugs (mexiletine, n=1; disopyramide, n=2; bepridil, n=5; quinidine, n=4).

The appropriate shock-free survival rate in the secondary prevention group was significantly lower than that in the primary prevention group (Figure 1).

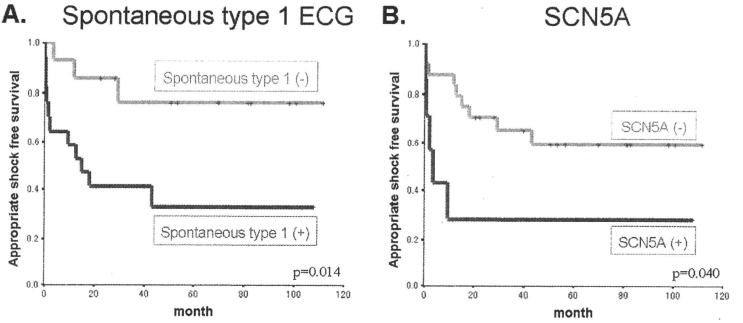


**Figure 3.** Appropriate-shock-free survival rate in Brugada syndrome (BS) patients with an implantable cardioverter defibrillator for secondary prevention. (A) 11 (64.7%) of 17 patients with spontaneous type 1 ECG and 3 (21.4%) of 14 patients without spontaneous type 1 ECG received appropriate shocks. The appropriate-shock-free survival rate was significantly lower in patients with spontaneous type 1 ECG than in BS patients without spontaneous type 1 ECG (41.1% vs 85.7% at 2 years, P=0.014). (B) 5 (71.4%) of 7 patients with a *SCN5A* mutation and 9 (37.5%) of 24 patients without *SCN5A* mutation received appropriate shocks. The appropriate-shock-free survival rate was significantly lower in patients with a *SCN5A* mutation (28.6% vs 83.3% at 1 year, P=0.040).

**Table 5. Characteristics of BS Patients With and Without *SCN5A* Mutation and Implanted ICD for Secondary Prevention**

| | ***SCN5A* mutation (+)** | ***SCN5A* mutation (–)** | **P value** |
|---|---|---|---|
| N | 7 | 24 | |
| Male, (%) | 6 (85.7) | 23 (95.8) | 0.338 |
| Age (years) | 47.0±14.6 | 47.8±10.8 | 0.440 |
| No. of appropriate shock | 6.6±6.2 | 1.7±3.0 | 0.007 |
| Period from ICD implantation to drug administration or last follow-up | 59.1±35.9 | 46.5±35.0 | 0.414 |
| Drug administration, (%) | 4 (57.1) | 7 (29.2) | 0.134 |
| Spontaneous type 1 ECG, (%) | 6 (85.7) | 11 (45.8) | 0.062 |
| Family history of SD, (%) | 0 (0) | 5 (20.8) | 0.187 |
| Documented VF, (%) | 5 (71.4) | 17 (70.8) | 0.976 |
| VF induced by programmed electrical stimulation, (%) | 1 (14.3) | 17 (70.8) | 0.008 |
| Late potential, (%) | 3/4 (75.0) | 17/24 (70.8) | 0.864 |
| QRS duration (ms) | 110±14 | 102±22 | 0.399 |
| HV interval (ms) | 52.2±12.8 | 43.5±6.5 | 0.204 |

Abbreviations see in Tables 1,2.

In the secondary prevention group, there were no significant differences in the appropriate ICD shock-free survival rate between patients with and without VF induced by PES, family history of SD and LPs (Figure 2). However, the appropriate shock-free survival rate in patients with spontaneous type 1 ECG (n=17) was significantly lower than that in patients without a spontaneous type 1 ECG (n=14) (Figure 3A). The appropriate shock-free survival rate in patients with *SCN5A* mutation (n=7) was also significantly loweer than that in patients without a mutation (n=24) (Figure 3B).

There was no significant difference in the number of appropriate ICD shocks between patients with and those without spontaneous type 1 ECG. However, appropriate ICD shock was more frequent in patients with a *SCN5A* mutation than in patients without during the period from ICD implantation to first drug administration (in patients with drugs) or last follow-up (in patients without drugs) (Table 5).

In the secondary prevention group, the odds ratio (OR) of spontaneous type 1 ECG for VF recurrence was 2.48 (P=0.248) at 1 year after ICD implantation, 5.24 (P=0.043) at 2 years after ICD implantation and 6.72 (P=0.021) at last follow-up. Also, the OR of *SCN5A* mutation for VF recurrence was 9.50 (P=0.021) at 1 year after ICD implantation, 5.00 (P=0.081) at 2 years after ICD implantation and 4.17 (P=0.128) at last follow-up.

### Clinical Course of BS Patients Without ICD

None of the BS patients without an ICD suffered from VF or SD during the follow-up.

## Discussion

### New Findings

In the present study, we demonstrated that *SCN5A* mutations are not associated with the first onset of VF in BS patients. However, they are associated with early and frequent recurrence of VF in symptomatic BS patients. To our knowledge, this is the first report on the genotype–phenotype interaction and clinical significance of *SCN5A* mutations.

### *SCN5A* Mutation Is Related to Depolarization Abnormality

In 1998, Chen et al[23] identified the first mutation in *SCN5A*, the gene encoding for the alpha subunit of the sodium channel ($I_{Na}$), that was linked to BS.[23] Mutations of *SCN5A* account for 10–30% of BS cases. Functional analysis using expression systems have revealed that mutations in *SCN5A* result in loss of function of $I_{Na}$. *SCN5A* has been reported as associated with depolarization abnormalities.[6,7,9–11] Previous studies showed that the P wave, QRS, PQ, and HV interval were significantly longer in BS patients with a *SCN5A* mutation than in those without.[7,9,10] Bradyarrhythmias, such as sick sinus syndrome and intraventricular conduction delay, are also more likely to occur in BS patients with *SCN5A* mutations.[10,11]

### *SCN5A* Mutation Is Not Related to Initial Episodes of VF

Detection of a *SCN5A* mutation is a diagnostic tool for BS. However, no study has shown the impact of genetic analysis on risk stratification. Priori et al reported that *SCN5A* mutation is the most frequent mutation in known causative genes, but the incidence is only 10–30%.[24] Furthermore, the average penetrance based on ECG analysis is only 16%.[25] Several studies have also shown that *SCN5A* mutation is not associated with clinical significance, such as syncopal or VF episodes.[15,16] The present study also showed that *SCN5A* mutation was not associated with initial VF episodes in BS patients.

### *SCN5A* Mutation Is Associated With Early and Frequent VF Recurrence

To our knowledge, only 1 study of mice with ischemic heart disease has reported an association of *SCN5A* mutation with recurrence of VF.[26]

In the present study, 5 (71.4%) of the 7 symptomatic BS patients with a *SCN5A* mutation had recurrence of VF within 1 year after ICD implantation. On the other hand, only 9 (37.5%) of the 24 symptomatic BS patients without *SCN5A* mutation had recurrence of VF up till the last follow-up. The recurrence of VF was significantly earlier and more frequent in symptomatic BS patients with *SCN5A* mutation than in symptomatic BS patients without a mutation.

The reasons why *SCN5A* mutation is associated with the clinical significance of symptomatic BS are speculative, based on some previous studies. Aiba et al reported that depolarization abnormalities in experimental BS models are associated with factors that predispose to VF maintenance.[27] Junttila et al also reported that prolonged QRS duration is associated with VF episodes.[16] Similar observations have been made in clinical settings where the occurrence of LPs in SAECG predicts adverse events in BS patients.[28] Yokokawa et al have also linked prolonged QRS duration and aging to *SCN5A* mutation carrier status in BS patients.[7] In the present study, SCN5A was also associated with prolonged QRS duration and HV interval. Thus, *SCN5A* mutation is associated with a prolonged and progressive conduction delay, which is strongly associated with VF. Therefore, *SCN5A* mutations might be the cause of early and frequent recurrence of VF.

### Clinical Implications

Management of symptomatic BS patients is very important because frequent ICD shocks are likely to worsen quality of life and cause psychological damage. Therefore, risk stratification of symptomatic BS patients is important. In the present study, we showed that having a *SCN5A* mutation was a predictor of early and frequent VF recurrence. Therefore, symptomatic BS patients with spontaneous type 1 ECG or *SCN5A* mutation should be administered drugs to avoid early and frequent recurrence of VF.

### Study Limitations

First, the mean follow-up period was not long, and it is necessary to evaluate the same data during a longer period. Second, the number of the BS patients with *SCN5A* was relatively low. If the number of BS patients increased, the results might change.

## Conclusions

We demonstrated that *SCN5A* mutation is not associated with initial episodes of VF in BS, but is associated with early and frequent recurrence of VF in symptomatic BS patients. To our knowledge, this is the first report of a genotype–phenotype interaction and the clinical significance of *SCN5A* mutation.


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## Disclosures

None.


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